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Characterization and Regulation of the *Pseudomonas aeruginosa algC* Gene Encoding Phosphomannomutase*

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The nucleotide sequence of the *Pseudomonas aeruginosa algC* gene encoding phosphomannomutase (PMM; EC 5.4.2.8) was determined. The codon usage in *algC* in the wobble base position was 90.4% G+C, typical of *Pseudomonas* genes. The predicted amino acid sequence of phosphomannomutase (PMM) showed homology over a stretch of 112 amino acids in the carboxyl terminus with rabbit muscle phosphoglucomutase (PGM), an enzyme that catalyzes a reaction analogous to that catalyzed by PMM. In addition, a specific amino acid sequence within PMM showed homology with the catalytic site of PGM. DNA sequence analysis of a defective *algC* gene (*algC'*) cloned from a mutant of *P. aeruginosa* that lacked PMM activity revealed one point mutation (a C to T transition) in the carboxyl terminus of PMM which resulted in an amino acid change from arginine 420 to cysteine 420. The mutation identified in the *algC'* gene was not within the regions of homology with PGM. The *algC* promoter showed significant homology with the promoters of two other *P. aeruginosa* genes involved in alginate synthesis, *algD* and *algR1*. Both the *algD* and *algR1* promoters are activated by the product of the *algR1* gene in *P. aeruginosa*. The upstream region of the *algC* gene contained a sequence identical to the *algD* upstream sequence that is known to be the binding site for the AlgR1 protein. Expression of *algC* was reduced 5.7-fold in an *algR1* mutant of *P. aeruginosa* compared to its isogenic parent strain (lacking the *algR1* mutation), suggesting that the *algR1* gene product activates the transcription of the *algC* gene.

One clinical manifestation of cystic fibrosis (CF)[1] is the production of large amounts of hyperviscous bronchial secretions. The accumulation of this material in the respiratory tract of CF patients appears to make these individuals especially vulnerable to bacterial lung infections. *Pseudomonas aeruginosa* is a prevalent pathogen in the lungs of CF patients (1). While *P. aeruginosa* can cause damage to the lung tissue by producing toxins and proteolytic enzymes, the primary complication resulting from *P. aeruginosa* infection in CF is the production by the bacterial cells of a slimy exopolysaccharide known as alginate (1). The presence of alginate exacerbates the respiratory difficulties resulting from the abnormally viscous CF lung environment.

The alginate layer surrounding *P. aeruginosa* in the CF respiratory tract provides a protective barrier against antibiotics and the host immune defenses (1, 2). Clearly, prevention of alginate synthesis by *P. aeruginosa* in the CF lung would enhance existing treatment strategies. Thus, compounds that inhibit enzymes required for alginate synthesis in *P. aeruginosa* (see Fig. 1) have potential use as therapeutic agents. The development of such drugs has been hindered by the inability to obtain sufficient quantities of alginate biosynthetic enzymes for characterization and inhibitor studies. This is due to the extremely low activity levels of these enzymes in cell-free extracts, even in those prepared from heavily mucoid (alginate-producing) *P. aeruginosa* strains (3). To circumvent this problem, many of the *P. aeruginosa* alginate (*alg*) genes have been cloned in broad host range-controlled expression vectors to allow overproduction of the gene product of interest. Two alginate biosynthetic enzymes from *P. aeruginosa*, PMI-GMP (the *algA* gene product), and GMD (the *algD* gene product) (see Fig. 1), have been overproduced and purified using this method (4–7). The gene encoding PMM (step 2, Fig. 1), was of interest as PMM represents yet another potential target of inhibition of alginate synthesis. In addition, although phosphomannomutases from yeast (8, 9) and plants (10) have been studied, very little information about PMM from bacterial sources has been reported in the literature. In this paper, we describe the cloning of the *P. aeruginosa algC* gene encoding PMM, present the nucleotide sequences of the wild-type and mutant *algC* genes as well as the transcriptional and translational initiation sites of the wild-type gene, and examine the transcriptional regulation of the *algC* gene in *P. aeruginosa*.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Cloning of a P. aeruginosa Gene Encoding PMM—The activity levels of PMM and other alginate biosynthetic en-

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[1] The abbreviations used are: CF, cystic fibrosis; PMM, phosphomannomutase; PMI-GMP, phosphomannose isomerase-GDP mannose pyrophosphorylase; GMD, guanosine diphospho-D-mannose dehydrogenase; Ap, ampicillin; Km, kanamycin; Tc, tetracycline; bp, base pair; kb, kilobase pair; EMS, ethyl methane sulfonate; PIA, *Pseudomonas* isolation agar; IPTG, isopropyl β-D-thiogalactopyranoside; MOPS, *N*-morpholinopropanesulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; PGM, phosphoglucomutase.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Tables I–III, and V, and Figs. 3–9) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

zymes in crude extracts of *P. aeruginosa* are extremely low and can barely be detected (3). One of our objectives was to clone the *P. aeruginosa algC* gene under *tac* promoter control to allow overproduction of PMM, thereby obtaining sufficient amounts of the enzyme for characterization and inhibitor studies. In order to achieve this objective, we first identified an alginate-negative mutant of *P. aeruginosa* that lacked PMM activity (strain 8858), using the strategy outlined under "Results" in the Miniprint. We then screened genomic libraries of *P. aeruginosa* 8830 for recombinant plasmids that would complement the *alg-28* mutation in mutant 8858, using the approach described under "Experimental Procedures." From a *Bam*HI-generated library, we recovered a recombinant plasmid (pAB8) that restored the mucoid (alginate-producing) phenotype to mutant 8858. The level of alginate produced by strain 8858 containing pAB8 was comparable to that observed for the mucoid parent strain 8830 (Table III).

Subcloning of various cloned fragments from within the 26-kb insert in pAB8 localized the ability to complement mutant 8858 back to the mucoid phenotype to a 2.6-kb *Hin*dIII-*Sst*I fragment. The plasmid containing this 2.6-kb fragment was designated pNZ15. The size of the 2.6-kb insert was further decreased by making unidirectional deletions from the *Hin*dIII end using exonuclease III. This resulted in a 1.8-kb fragment that complemented mutant 8858 but only when cloned downstream of a vector-encoded promoter sequence (pNZ15-Δ2C). All of the various plasmids that contained subcloned fragments of the original 26-kb insert in pAB8 and still complemented mutant 8858 were passaged through *Escherichia coli* AC80. Upon reintroduction into 8858 these plasmids again restored the mucoid phenotype. We demonstrated previously (29) by hybridization analysis that the cloned 2.6-kb insert in pNZ15 was of *P. aeruginosa* origin. This fragment hybridized with a 2.6-kb fragment of *Hin*dIII-*Sst*I-digested chromosomal DNA from *P. aeruginosa* strains 8830 and PAO1, as well as with DNA fragments of varying sizes from several other *Pseudomonas* species belonging to *Pseudomonas* rRNA homology group I, *Azomonas macrocytogenes*, *Azotobacter vinelandii*, *Serpens flexibilis*, and *Xanthomonas campestris* (29). The fragment did not hybridize with *Hin*dIII-*Sst*I-digested chromosomal DNA from *E. coli*, *Salmonella typhimurium*, or *Klebsiella pneumoniae* (29).

Identification of P. aeruginosa Proteins Encoded by the 2.6-kb Insert in Plasmid pNZ15—In order to determine the number and size(s) of polypeptides encoded by the cloned *P. aeruginosa* DNA in plasmid pNZ15, and the direction of transcription of the putative *P. aeruginosa algC* gene, the 2.6-kb *Hin*dIII-*Sst*I fragment was cloned into the vectors pT7-5 and pT7-6 (16, 17). This allowed exclusive labeling of the plasmid-encoded polypeptides using a T7 RNA polymerase/plasmid promoter system in *E. coli* strain K-38/pGP1-2 (16, 17) (see "Experimental Procedures"). The resulting hybrid plasmids, pNZ530 and pNZ631, contain the cloned *Hin*dIII-*Sst*I fragment of *P. aeruginosa* DNA in opposite orientations relative to the T7 RNA polymerase promoter (Fig. 2). In these vectors, the β-lactamase gene is not selectively transcribed. The plasmids were introduced into *E. coli* strain K-38/pGP1-2 and plasmid-encoded proteins were selectively labeled with [^{35}S]methionine following growth, heat induction, and rifampicin treatment of the strains (16, 17). The results shown in Fig. 2 indicate that a single polypeptide having an estimated molecular weight of 50,000 was synthesized from the cloned insert in plasmid pNZ631. This polypeptide was formed even in the absence of thermal induction, demonstrating incomplete repression of the T7 RNA polymerase at 30 °C in these strains. No additional polypeptides were synthesized in plasmid pNZ530 compared to the vector control pT7-5, suggesting that the direction of transcription of the putative *algC* gene is from *Hin*dIII to *Sst*I in the 2.6-kb cloned insert in pNZ15.

Enzymological Analyses—Plasmids containing the putative *P. aeruginosa algC* gene, either on the aforementioned 2.6-kb *Hin*dIII-*Sst*I fragment or its 1.8-kb deletion derivative were introduced into strain 8858 to test for increased PMM activity (Table IV). Strain 8858 alone had extremely low levels of PMI and PMM activity. The presence of plasmid pNZ15 in strain 8858 led to a high level of PMM activity. When the

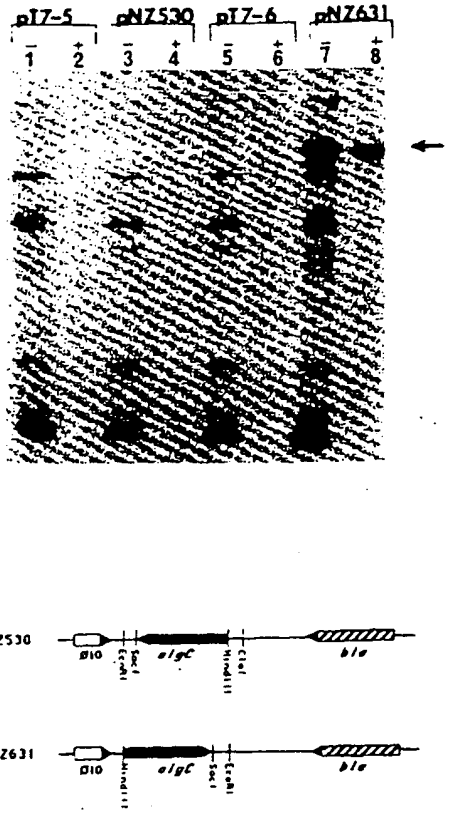

FIG. 2. **Autoradiogram showing the single polypeptide encoded by the cloned 2.6-kb *Hin*dIII-*Sst*I DNA fragment that complements *P. aeruginosa* mutant 8858.** A + indicates thermal induction (42 °C for 10 min). A − indicates no induction. Cells were treated with rifampicin prior to ^{35}S-labeling of proteins. After SDS-PAGE, radioactive polypeptides were visualized by autoradiography. *Lanes 1* and *2*, pT7-5 vector control; *lanes 3* and *4*, pNZ530 (2.6-kb *Hin*dIII-*Sst*I fragment cloned into pT7-5 with the *Sst*I site proximal to the T7 promoter); *lanes 5* and *6*, pT7-6 vector control; *lanes 7* and *8*, pNZ631 (2.6-kb *Hin*dIII-*Sst*I fragment cloned into pT7-6 with the *Hin*dIII site proximal to the T7 promoter). The position of PMM is indicated by the *arrow*. The position of the cloned inserts relative to the T7 promoter in each plasmid is represented schematically in the bottom of the figure.

FIG. 1. **Alginate biosynthetic pathway in *P. aeruginosa*.** Abbreviations: *F6P*, fructose 6-phosphate; *M6P*, mannose 6-phosphate; *M1P*, mannose 1-phosphate; *GDPM*, GDP-mannose; *GMA*, GDP-mannuronic acid; *PMI*, phosphomannose isomerase; *PMM*, phosphomannomutase; *GMP*, GDP-mannose pyrophosphorylase; *GMD*, GDP-mannose dehydrogenase. The *P. aeruginosa algA* gene encodes a bifunctional PMI-GMP (7), *algD* encodes GMD (4), and *algC* encodes PMM (this report). Steps between GMA and alginate include polymerization, epimerization, acetylation, and export, although little information is currently available about these steps.

TABLE IV
Specific activities of PMI and PMM in P. aeruginosa 8858 and E. coli AC80 containing subclones derived from pAB8

Strain/plasmid[a]	Specific activity			
	PMI		PMM	
	-IPTG	+IPTG	-IPTG	+IPTG
	milliunits/mg			
P. aeruginosa				
8858	<1.0[b]	<1.0	<1.0[b]	<1.0
8858/pNZ15	<1.0	NT[c]	93.6	NT
8858/pMMB22	<1.0	<1.0	<1.0	<1.0
8858/pNZ18	<1.0	<1.0	54.7	88.2
8858/pMMB66HE	<1.0	<1.0	<1.0	<1.0
8858/pNZ48	<1.0	<1.0	51.9	38.8
8858/pNZ49	<1.0	<1.0	156.3	325.0
E. coli				
AC80	202.4[b]	NT	87.9[b]	NT
AC80/pMMB66HE	225.1	170.5	86.3	NT
AC80/pNZ49	172.2	239.6	76.4	76.2

[a] Plasmid pNZ15 contains the *algC* gene cloned as a 2.6-kb fragment in pJRD215 (23). Plasmid pNZ18 contains the same 2.6-kb fragment cloned under *tac* promoter control in plasmid pMMB22 (44). Plasmids pNZ48 and pNZ49 contain the *algC* gene (cloned as a 1.8-kb fragment under *tac* promoter control) in plasmids pMMB66EH and pMMB66HE (30), respectively.

[b] A specific activity of <1.0 for PMI represents the wild-type level for *P. aeruginosa* (3). The endogenous levels of PMI and PMM activity shown for *E. coli* AC80 are typical for this strain grown under these conditions (3).

[c] Not tested as the *algC* gene is not under *tac* promoter control.

2.6-kb *Hin*dIII-*Sst*I fragment was cloned in the proper orientation under *tac* promoter control (plasmid pNZ18), a high level of PMM activity was also observed. This activity did not exceed that obtained with plasmid pNZ15, and the activity increased only moderately upon IPTG induction (Table IV). DNA sequencing of the 2.6-kb fragment cloned in pNZ15 (see below) indicated that the single open reading frame in this fragment lies approximately 1.2 kb from the *Hin*dIII end of the fragment. Thus, it appeared that the inability to overexpress the putative *algC* gene in pNZ18 was due to the 1.2 kb of intervening DNA between the *tac* promoter and the start of the *algC* gene. To test this possibility, the 1.8-kb deletion derivative of the 2.6-kb *Hin*dIII-*Sst*I fragment was cloned in both orientations relative to the *tac* promoter in the vectors pMMB66EH and pMMB66HE (30) to create plasmids pNZ48 and pNZ49, respectively. The latter plasmids were introduced into *P. aeruginosa* strain 8858 and crude extracts were prepared from cultures grown with and without IPTG induction (Table IV). Although a high level of PMM activity was observed in 8858 containing pNZ48, the activity did not exceed that observed for plasmid pNZ18, and the PMM activity did not increase upon IPTG induction. Strain 8858/pNZ49 yielded the highest PMM activity observed for any strain/plasmid combination, and this PMM activity increased 2.1-fold upon IPTG induction. The direction of transcription of the putative *algC* gene relative to the *tac* promoter in pNZ49 was the same as that in plasmid pNZ631, the only construct that directed the synthesis of a ^{35}S-labeled protein using the T7 RNA polymerase/plasmid promoter system (see Fig. 2). No increase in PMI activity was observed in any *P. aeruginosa* strain harboring a plasmid containing the putative *algC* gene, and no PMM (or PMI) activity was found associated with the plasmid vectors alone (Table IV).

E. coli contains high endogenous levels of PMI and PMM activity (3, Table IV). Introduction of plasmid pNZ49 did not lead to a significant difference in the level of PMM (or PMI) activity in crude extracts of *E. coli*, even upon IPTG induction (Table IV). However, when a crude extract of *E. coli* AC80/pNZ49 (prepared from IPTG-induced cells) was fractionated by ion-exchange chromatography, a second peak of PMM activity was observed in addition to the peak of PMM activity endogenous to *E. coli* (Fig. 3).

Samples of the crude extracts prepared from *P. aeruginosa* 8858 and *E. coli* AC80 overexpressing the putative *P. aeruginosa algC* gene, along with the appropriate vector and uninduced controls, were subjected to SDS-PAGE (Fig. 4). The *P. aeruginosa* strains having elevated PMM activity, 8858/pNZ18 and 8858/pNZ49, contained a protein band corresponding to an estimated molecular weight of 51,000. This was in agreement with the molecular weight (50,000) of the single ^{35}S-labeled polypeptide synthesized from the 2.6-kb *Hin*dIII-*Sst*I fragment in plasmid pNZ631 (Fig. 2). *E. coli* contained numerous intense protein bands that comigrated to the position corresponding to a molecular weight of 51,000 (see Fig. 4). Thus, it was not possible to discern an additional band encoded by the putative *P. aeruginosa algC* gene in *E. coli* AC80/pNZ49 even with IPTG induction. Likewise, when samples of column fractions spanning both peaks of PMM activity from *E. coli* AC80/pNZ49 (+IPTG) recovered from ion-exchange chromatography (see Fig. 3) were subjected to SDS-PAGE, numerous intense protein bands in the M_r 51,000 range obscured any band that may have been encoded by the cloned insert in pNZ49 (data not shown).

To confirm that the cloned DNA fragment in pNZ49 contained the *algC* structural gene encoding the PMM polypeptide, PMM from *P. aeruginosa* 8858/pNZ49 was purified to an extent that allowed its N-terminal amino acid sequence to be determined (see "Experimental Procedures"). Following sequential purification steps using ion-exchange, hydroxylapatite, and gel-filtration chromatography (Fig. 5), PMM was judged to be >90% pure based on SDS-PAGE (Fig. 6). The N-terminal amino acid sequence of the protein, read to 19 amino acids, was S-T-V-K-A-P-T-L-P-A-S-I-F-R-A-Y-D-I-R. This sequence corresponded exactly to the N-terminal amino acid sequence predicted from the nucleotide sequence of the *algC* gene (described below).

DNA Sequence Analysis—The complete nucleotide sequence of the *P. aeruginosa algC* structural gene contained within the 1.8-kb DNA fragment that complemented mutant 8858 (described above) was determined (Fig. 7). One open reading frame was identified which was capable of coding for a protein of 463 amino acids. The calculated molecular weight of PMM predicted from the nucleotide sequence of the *algC* gene (50,269) was in agreement with the molecular weight of PMM (51,000) determined by SDS-PAGE. The codon usage in the wobble base position was 90.4% G+C (see Table V), typical of *Pseudomonas* genes (31, 32). The direction of transcription of *algC* was shown (Fig. 2) to be from the *Hin*dIII site to the *Sst*I site of the 2.6-kb fragment in plasmid pNZ15. Following determination of the N-terminal amino acid sequence of PMM (described above), it was determined that translation of the *algC* message initiated with a methionine and terminated with a TGA codon 1,388 nucleotides downstream of the ATG (Fig. 7). A putative ribosome-binding sequence, GGAG (33), was located 12 bp upstream of the translational initiation codon (Fig. 7). The 5′ end of the *algC* mRNA was found to be a G located 244 bp upstream of the translational initiation site (data shown below).

A sequencing strategy analogous to that used for the wild-type *algC* gene was used to sequence the *algC′* gene cloned from the PMM$^-$ mutant *P. aeruginosa* 8858 (as described under "Experimental Procedures"). A single C to T transition was found at nucleotide position 1505 of the *algC′* coding

region, resulting in a change from arginine 420 to cysteine 420 (data not shown).

Comparison of the nucleotide sequence of *algC* with other nucleotide sequences in GenBank®/EMBL Data Bank (34) showed only weak overall homologies. However, PMM did show significant localized amino acid homology with phosphoglucomutase (PGM) from rabbit muscle (35). PMM and PGM catalyze analogous interconversions of hexose 6-phosphates and hexose 1-phosphates. The 44% sequence similarity was restricted to a 112 amino acid stretch located in the carboxyl terminus of both enzymes (Fig. 8). The significance of the homology in this region is not known at present.

PGM belongs to a family of phosphoserine enzymes which share a serine residue that is phosphorylated in the active form of the enzyme (36). The amino acid sequence Thr-Ala-Ser-His-Asn is known to be critical for PGM activity, and this sequence is within the 21 amino acid long active site region of PGM (35). The amino acid sequence Thr-Gly-Ser-His-Asn was found in the *algC* coding sequence (amino acid positions 106–110, Fig. 7). The region surrounding the Thr-Gly-Ser-His-Asn sequence in PMM was compared with the 21 amino acid long active site region of PGM (Fig. 9). Considerable homology (57%) between PMM and PGM was observed in this region (which lies in the N-terminal portion of both proteins). Furthermore, of the matched amino acids comprising this 57% homology, 75% (9 out of 12) were exact matches (only 25% of the matches were conserved replacements, see Fig. 9).

Identification of the Transcriptional Initiation Site of algC—The 5′ end of the *algC* gene was mapped using S1 nuclease protection (24). A 15-bp synthetic oligonucleotide (5′GCGGAAGATGCTGGC3′) designated PE1 was used as primer. PE1 is complementary to nucleotides 275–290 of the *algC* DNA sequence (Fig. 7). A 1.5-kb *Hin*dIII-*Xho*I DNA fragment from within the 2.6-kb DNA fragment in plasmid pNZ15 contains the putative *algC* promoter region and an additional 1 kb of upstream *P. aeruginosa* DNA. This fragment was cloned into the *Hin*dIII-*Sal*I sites of M13mp19 resulting in phage mNZ25 which was used as the source of single-stranded DNA. The 1.5-kb *Hin*dIII-*Xho*I fragment was hybridized to total cellular RNA from the mucoid *P. aeruginosa* strain 8830 and to RNA from a nonmucoid strain of *P. aeruginosa* (8822) harboring plasmid pNZ15-ΔHE, which contains the *algC* structural gene and 1.2 kb of *P. aeruginosa* DNA upstream of the translational start site of *algC* (the promoter of the vector-encoded tetracycline resistance gene was deleted in plasmid pNZ15-ΔHE, see below). Analysis of the protected DNA fragment(s) on a low resolution polyacrylamide gel revealed one strong signal migrating to a position corresponding to 274 bp in length (Fig. 10). Since the same primer was used for the S1 mapping and the dideoxy sequence analysis, transcription was determined to initiate with a guanosine located 244 bp upstream of the *algC* translational initiation codon.

A comparison of the upstream region preceding the *algC* structural gene with the consensus *E. coli* RNA polymerase σ^{70} recognition sequence revealed no similarity in the −10 or the −35 regions. We did observe that the GC at positions −21 and −22 and the GG at positions −33 and −34 of the *algC* gene had the same spacing as the GC at positions −12 and −13 and the GG at positions −24 and −25 of an enteric bacterial *rpoN* (σ^{54}) consensus sequence (37, 38) (Fig. 11). However, the position of the bases relative to the transcriptional start site was different in the *algC* sequence and the *rpoN* consensus sequence. The significance of this shift in position is not known at present. More importantly, perhaps, was the finding that the GC and GG sequences at positions

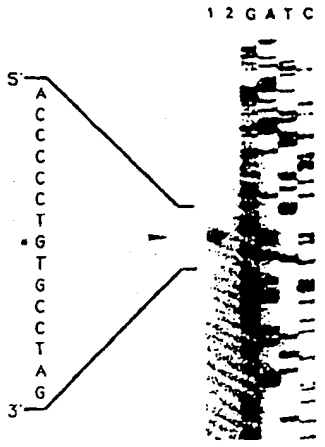

FIG. 10. **Identification of the transcriptional initiation site of *algC*.** S1 nuclease mapping was performed with a 15-mer synthetic oligonucleotide (PE1) which was complementary to the sense strand of the 5′ end of *algC*. The probe was hybridized to total cellular RNA from *P. aeruginosa* strain 8830 (*lane 1*) and *P. aeruginosa* strain 8822 harboring the plasmid pNZ15-ΔHE (*lane 2*). Products of the S1 nuclease reactions are adjacent to dideoxy sequencing reaction lanes (*GATC*) prepared with primer PE1. The complementary sequence is shown to the left of the sequencing reaction. The *arrow* and *asterisk* indicate the guanosine corresponding to the products protected from S1 nuclease. This position is 244 bp upstream of the translational start site (see Fig. 7).

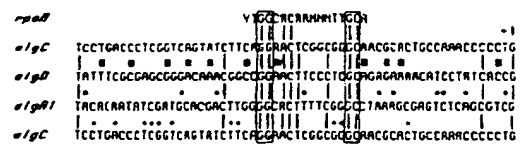

FIG. 11. **Comparison of the sequence of the putative promoter region of *algC* with that of *algD*, *algR1*, and the σ^{54} (RpoN) consensus sequence.** The transcriptional start site is designated as +1. The GG and GC within the putative σ^{54} recognition sequence are *boxed*. Identical base matches between *algC* with *algD*, *algR1*, and the *rpoN* consensus are indicated by *solid vertical lines*. Identical bases between *algC* and *algD* are indicated by ■, between *algD* and *algR1* by ●, and between *algR1* and *algC* by ◆. Other letters are defined as follows: *Y*, pyrimidine; *R*, purine; and *N*, any base.

−21/−22 and −33/−34 of *algC* also exist at these same positions in the upstream region of two other *P. aeruginosa* genes involved in alginate synthesis, *algD* and *algR1* (39, 40) (Fig. 11).

Analysis of algC Gene Expression—To construct plasmid pNZ15, the 2.6-kb *Hin*dIII-*Sst*I fragment containing the *algC* structural gene was cloned between the *Hin*dIII-*Sst*I sites of pJRD215, which placed the *algC* gene downstream of the tetracycline resistance gene promoter present in pJRD215 (23). To determine if the *algC* gene was being expressed from this vector-specific promoter or from its own promoter, the *Eco*RI-*Hin*dIII vector segment containing the *tet* promoter was removed from pNZ15, generating plasmid pNZ15-ΔHE. This construct was still able to complement the alginate-negative *P. aeruginosa* mutant 8858, indicating that the *algC* gene within the 2.6-kb fragment was indeed being expressed from its own promoter.

The *P. aeruginosa algD* gene is transcriptionally activated in mucoid strains of *P. aeruginosa* (4). This activation is mediated by the product of the *algR1* gene, a *trans*-acting regulatory protein that autoregulates the expression of *algR1* itself as well as activating *algD* (40). Since the upstream region of the *algC* gene showed similarity both in sequence and spacing to the upstream regions of both the *algD* and *algR1*

TABLE VI
β-Galactosidase activities in P. aeruginosa strains containing the algC-lacZ transcriptional fusion

Strain/plasmid[a]	β-Galactosidase specific activity[b]
8830/pNZ63	51
8852/pNZ63	9
8858/pNZ63	200

[a] Cells were grown for 18 h in YTG medium (see "Experimental Procedures"). The doubling time (2 h) was the same for all strains in this medium.

[b] Specific activities are defined as nanomoles of *o*-nitrophenol formed/minute/milligram of crude extract protein. The specific activity values given are the average of three independent experiments that gave essentially identical results.

genes (Fig. 11), it was possible that the *algC* gene was also transcriptionally regulated by the *algR1* gene product. To test this possibility, a transcriptional fusion vector was constructed using the *algC* upstream region from pNZ15 (1.5 kb of DNA upstream of the internal *Xho*I site at nucleotide position 533, see Fig. 7) and the promoterless *lacZ* structural gene in the vector pKRZ-1, a broad host-range promoter probe vector based on the pSA origin of replication.[3] This placed the promoterless *lacZ* gene directly under the control of the *algC* promoter sequence and 1.0 kb of upstream DNA, creating plasmid pNZ63. Plasmid pNZ63 was introduced into the stable mucoid (alginate-producing) *P. aeruginosa* strain 8830 and an *algR1* mutant derived from 8830 (strain 8852), to determine if transcriptional activation of *algC* was dependent on a functional *algR1* gene. *E. coli* CSH50 (25) containing pNZ63 was also examined as a negative control. In three independent experiments, the specific activity of β-galactosidase was 4.8–6.6-fold (average 5.7-fold) lower in 8852/pNZ63 compared to 8830/pNZ63 grown under the same conditions (Table VI). Negligible levels of β-galactosidase activity were detected in *E. coli* CSH50/pNZ63.

PMM activity is induced in *P. aeruginosa* in response to overproduction of the preceding enzyme of the alginate biosynthetic pathway, phosphomannose isomerase (3). This suggested that the product of the PMI reaction, mannose 6-phosphate, may be involved in the induction of PMM activity. Since *P. aeruginosa* strain 8858 lacks PMM activity, it would be expected to accumulate mannose 6-phosphate as a result of the mutational block at PMM. If mannose 6-phosphate was indeed involved in inducing PMM activity by increasing the level of *algC* transcription, then strain 8858 should have high levels of *algC* transcription, even though the message formed coded for an inactive PMM. To test this possibility, we introduced the *algC-lacZ* fusion vector pNZ63 into strain 8858 and compared the level of β-galactosidase activity to strain 8830 (from which 8858 was derived) (11) containing pNZ63. The level of *algC* transcription, measured as β-galactosidase activity, was 4-fold higher in strain 8858/pNZ63 than in 8830/pNZ63 (Table VI).

DISCUSSION

The inherently low activity levels of alginate biosynthetic enzymes in mucoid strains of *P. aeruginosa* (3, 41) have made it difficult to attribute the loss of alginate production in Alg⁻ mutants to a loss of a specific enzymatic activity. Also, such low enzyme activities hinder the purification of sufficient quantities of enzymes needed for rigorous characterization for development of inhibitors that have potential therapeutic application in the treatment of *P. aeruginosa* respiratory tract infections in CF patients. Our laboratory successfully cloned and overexpressed two *P. aeruginosa alg* genes, *algA* and *algD*, and overproduced and purified their respective gene products, PMI-GMP and GMD (4–7). In this report we describe the cloning, sequencing, and genetic characterization of yet another *P. aeruginosa alg* gene, *algC* (encoding PMM), and examine its transcriptional regulation.

We used an indirect approach to identify an Alg⁻ mutant of *P. aeruginosa* that lacked PMM activity. Sa'-Correia *et al.* (3) showed that overproduction of PMI in *P. aeruginosa* led to a simultaneous increase in PMM activity. By overexpressing the *algA* gene in several Alg⁻ mutants of *P. aeruginosa*, we were able to show that strain 8858 (*his-1*, *alg-28*) lacked the increase in PMM activity that normally accompanies overproduction of PMI, and thus appeared to have a mutation in the *algC* structural gene. Wang *et al.* (28) showed that none of the *alg* genes cloned previously in this laboratory encoded PMM, and it is interesting that strain 8858 belonged to a group of Alg⁻ mutants that were not complemented by any of the cloned *alg* genes in our collection. It was possible, however, that the mutation in strain 8858 resided in a regulatory gene that was involved in the induction of PMM activity. The loss of such a regulatory gene would be manifested in *P. aeruginosa* as an Alg⁻ phenotype and in an absence of elevated PMM activity in response to overproduction of PMI, characteristics indistinguishable from an *algC* structural gene mutant.

Strong evidence in favor of strain 8858 containing a mutation in the *algC* structural gene came from cloning of a *P. aeruginosa* gene that complemented the *alg-28* mutation and restored the mucoid phenotype to strain 8858. Plasmid pNZ49 contained the putative *algC* gene cloned under control of the *tac* promoter. Introduction of pNZ49 into strain 8858 led to highly elevated PMM activity that increased still further (2.1-fold) upon IPTG induction (Table IV). In addition, when plasmid pNZ49 was introduced into *E. coli* AC80 and the putative *algC* gene overexpressed by IPTG induction, two peaks of PMM activity were resolved following ion-exchange chromatography of crude extracts. Only the larger trailing peak of PMM activity shown in Fig. 3 is normally present in extracts of *E. coli* AC80. It is very unlikely that a *P. aeruginosa* regulatory gene that induces PMM activity in *P. aeruginosa* could cause induction of a previously unrecognized second isozyme of PMM in *E. coli*.

Taken together, these data suggested that the gene cloned in pNZ49 was the *P. aeruginosa algC* structural gene encoding PMM. However, proof of this could only be obtained by determining the N-terminal amino acid sequence of purified PMM and comparing it to the N-terminal amino acid sequence predicted from the nucleotide sequence of the *algC* gene. We have confirmed that the cloned gene in pNZ49 does in fact encode PMM, based on 100% agreement between the first 19 N-terminal amino acids in the purified PMM and the N-terminal sequence predicted from the nucleotide sequence of the *algC* gene. To our knowledge this is the first report of the cloning and sequencing of a gene encoding PMM from any organism.

The sequence similarity between the 21 amino acid long active site region in the N-terminal portion of PGM (35) and the region surrounding the amino acid sequence Thr-Gly-Ser-His-Asn in the N-terminal end of PMM (Fig. 9) suggests that this region may contain the PMM active site. Furthermore, the similarity in this region of the two proteins suggests that in PMM, like PGM (36), the serine residue within the active site may need to be phosphorylated to produce an active enzyme. Investigations into that possibility are underway using oligonucleotide-directed site-specific mutagenesis to

[3] R. Rothmel, manuscript in preparation.

identify regions of the *algC* gene that are important in forming the active site of PMM. The significance of the 44% sequence similarity in the carboxyl end of PMM and PGM is not known at present as the functional role of this region in either protein, if indeed one exists, is not known.

The nucleotide sequence of a mutant *algC* gene (*algC'*) cloned from the PMM⁻ mutant *P. aeruginosa* 8858 was also determined. One point mutation (a C to T transition) was detected at nucleotide position 1505, which results in an amino acid change of arginine 420 to cysteine 420. Both the *algC* and *algC'* genes were cloned behind the *tac* promoter in the proper orientation and overexpressed in *P. aeruginosa* 8858. Analysis by SDS-PAGE showed that both extracts contained one overproduced protein band corresponding to a subunit molecular weight of 51,000, the size of the wild-type PMM.[4] Since DNA sequencing of the *algC'* gene did not reveal any nonsense mutations, and since the *algC* and *algC'* genes appear to code for proteins of the same molecular weight, we conclude that the *algC'* gene cloned from the PMM⁻ mutant 8858 does not encode a truncated PMM. The mutation in the *algC'* gene is not within the putative active site sequence of PMM (Fig. 9) or in the region of homology with PGM (Fig. 8). Thus, the mechanism by which the arginine 420 to cysteine 420 mutation in the *algC'* gene abolishes PMM activity in mutant 8858 is not known at present.

Once the transcriptional initiation site for the *P. aeruginosa* *algC* gene was determined, the promoter region was compared to other prokaryotic promoters to determine which nucleotides may be important in RNA polymerase recognition. We found no sequence in the *algC* promoter region that resembled the *E. coli* consensus σ^{70} promoter sequence. This may explain the absence of β-galactosidase activity when pNZ63 (the *algC* promoter-*lacZ* transcriptional fusion vector) was present in *E. coli* CSH50. Although we did observe that the GC and the GG at positions −21/−22 and −33/−34, respectively, of the *algC* upstream region had the same spacing as the GC (−12/−13) and GG (−24/−25) of an *rpoN* consensus sequence, the difference in position and intervening sequence (see Fig. 11) makes it impossible to make any conclusions with respect to a possible functional parallel between the two promoters. However, the considerable homology between the *algC* promoter and the promoter sequences of two other *P. aeruginosa* genes involved in alginate synthesis, *algD* and *algR1* (see Fig. 11) is worthy of mention. The 1.8-kb DNA fragment that contains the entire *algC* coding region, the transcriptional initiation site, and 67 bases upstream of the *algC* transcriptional initiation site does not complement the PMM⁻ mutant 8858 unless cloned downstream of a plasmid promoter sequence. This suggests that there are bases further upstream of the putative *algC* promoter sequence that are crucial for initiating transcription. Both the *algD* and *algR1* genes of *P. aeruginosa* are activated by the *algR1* gene product AlgR1 (40), and AlgR1 has recently been demonstrated by DNA footprinting experiments to bind at the −376 to −389 and −452 to −465 14-mer sites having the sequence $CCGTTCGTCN_5$. This sequence is upstream of the *algD* transcription initiation site. This far upstream sequence is essential for activation of the *algD* promoter (42). Interestingly, the sequence CCGTTCGTC was also found in the upstream region of *algC* (nucleotides −86 to −94, see Fig. 7). The presence of this sequence, together with the homology between the putative promoter of the *algC* gene and the *algD* and *algR1* promoters, suggested that transcription of *algC*, *algD*, and *algR1* may be controlled by similar regulatory mechanisms, requiring *trans*-activation by AlgR1. We tested this possibility by introducing the *algC-lacZ* transcriptional fusion vector pNZ63 into the mucoid *P. aeruginosa* strain 8830 and its isogenic *algR1* mutant strain 8852 (40). The 5.7-fold lower level of β-galactosidase activity in 8852/pNZ63 compared to 8830/pNZ63 (Table VI) suggested that the *algR1* gene product does in fact play a role in the transcriptional regulation of *algC*. It is noteworthy that AlgR1 activates at least two alginate genes mapping at different positions on the *P. aeruginosa* genome; *algD* at 34 min (40) and *algC* (map position unknown but distinct from *algD*), and that the consensus AlgR1-binding sequence $CCGTTCGTCN_6$ has been located in the far upstream region of both of these genes. Further experiments are in progress to elucidate the regulatory mechanisms governing *algC* gene expression and to determine the overall role of *algR1* in regulating alginate biosynthesis in *P. aeruginosa*.

[4] N. A. Zielinski, unpublished results.

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SUPPLEMENTARY MATERIAL TO

"Characterisation and Regulation of the Pseudomonas aeruginosa algC Gene Encoding Phosphomannomutase"

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EXPERIMENTAL PROCEDURES

Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table I. P. aeruginosa 8821 is a mucoid (alginate-producing) CF isolate; strain 8822 is a spontaneous non-mucoid (alginate-negative) mutant derived from 8821 (11). Strain 8830 is a stable alginate-producing mutant obtained via EMS mutagenesis of strain 8822 (11). Alginate-negative mutants of P. aeruginosa were derived from strain 8830 by further EMS mutagenesis and were divided into alg complementation groups based on the ability of different recombinant plasmids carrying DNA fragments from a genomic library of 8830 to complement the alg mutations and restore the mucoid phenotype to a given set of mutants (11, 12). Mutants lacking an alg complementation group designation were not complemented by any of the recombinant plasmids tested previously (11, 12) and represent other alginate genes yet to be cloned.

Media and Culture Conditions

All media were prepared in distilled deionized water. Liquid cultures were grown in L broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter of water) or YTG (10 g tryptone, 3 g yeast extract, 2 g glucose per liter of water). Solid media used were L broth solidified with 1.5% agar (for E. coli) and PIA (13) (for P. aeruginosa). All cultures were grown at 37°C, and liquid cultures were aerated by shaking at 250 rpm. Antibiotic concentrations used for plasmid-containing strains were as follows: ampicillin (E. coli), 75 µg/ml; carbenicillin (P. aeruginosa), 350 µg/ml; tetracycline, 10 µg/ml and 100 µg/ml for E. coli and P. aeruginosa, respectively; kanamycin, 75 µg/ml and 1.2 mg/ml for E. coli and P. aeruginosa, respectively. Cells to be used to prepare cell-free extracts for β-galactosidase assays were grown for 18 h in YTG medium. Cells to be used for other enzyme assays as well as for purification of PMM were grown in L broth for a total of 6 h. Inocula were 1% volumes of overnight seed cultures. For overexpression of genes under control of the tac promoter, IPTG (final concentration, 1 mM) was added to cultures after 3 h of growth. Following completion of the growth period, cells were harvested by centrifugation, washed twice with 0.9% NaCl, and stored as frozen pellets at -70°C.

Genetic Procedures

Recombinant plasmids were introduced into P. aeruginosa strains by triparental matings using the helper plasmid pRK2013 (14). A BamHI-generated genomic library of P. aeruginosa 8830, constructed in the broad host-range cosmid cloning vector pCP13 and maintained in E. coli strain AC80 (43), was used to clone the P. aeruginosa algC gene by mating the E. coli cells containing the P. aeruginosa genomic library en masse into the P. aeruginosa alginate-negative mutant 8858 (his-1 alg-28) and simultaneously screening for tetracycline resistance and complementation of the alg-28 mutation (i.e., restoration of the mucoid phenotype). Mucoid colonies were purified and the plasmids subjected to further analysis (described in Results). Transformations, plasmid isolations, SDS-PAGE and agarose gel electrophoresis, and general cloning procedures were carried out using standard techniques (15).

Polypeptides encoded by cloned DNA fragments in recombinant plasmids were identified using an exclusive labelling T7 RNA polymerase/plasmid promoter system (16, 17). Fragments containing the P. aeruginosa algC gene were cloned in opposite orientations relative to the T7 RNA polymerase promoter in the vectors pT7-5 and pT7-6. This allows overexpression of algC from the T7 RNA polymerase promoter. The resulting plasmids, pNZ530 and pNZ631, were introduced into E. coli K-38/pGP1-2. Plasmid pGP1-2 contains the heat inducible T7 RNA polymerase gene (16, 17). Proteins encoded by pNZ530 and pNZ631 were selectively labelled by addition of 35S-labelled methionine to the aforementioned plasmid-containing strains following growth, heat induction (to induce the T7 RNA polymerase gene on pGP1-2), and rifampicin treatment as outlined by Ries et al. (17). Following exposure to 35S-labelled methionine, the cells were centrifuged and the pellet was suspended in denaturing buffer (17) to lyse the cells. The lysed suspensions were then subjected to SDS-PAGE and the radioactive proteins in the gel were visualized by autoradiography as previously described (17). The molecular weights of the proteins were estimated by comparison with the migration positions of 14C-labelled protein molecular weight standards run in adjacent lanes on the gel.

RNA used for S1 nuclease mapping was isolated from exponentially growing E. coli and P. aeruginosa cells. The strains were grown in 100 ml of L broth. Inocula (1%) were from overnight cultures. RNA was isolated using the isoguanidinium thiocyanate and phenol method as previously described (18). The RNA concentration was determined spectrophotometrically at 260 nm.

The dideoxynucleotide chain termination method (19) was modified and used for DNA sequence determination using Sequenase™ DNA polymerase as recommended by the manufacturer (at 37°C) and Klenow DNA polymerase (at 45°C). For all reactions 7-deaza-dGTP was substituted for dGTP to reduce electrophoretic band compression artifacts (20). The host strain used was E. coli JM109 (21). Preparation of sequencing gels, electrophoresis conditions, and autoradiography were performed as described previously (22) except that prior to autoradiography, gels were fixed in a 10% acetic acid:10% methanol solution for 10 min, transferred to Whatman 3MM paper, and dried.

A defective algC gene, designated algC', was also cloned for nucleotide sequencing in order to identify the nature of the mutation causing a loss of PMM activity. Genomic DNA from the P. aeruginosa alginate-negative mutant 8858 (which lacks PMM activity, see Results) was digested to completion with HindIII and SalI and separated on a 0.7% agarose gel. The region corresponding to 2.2-3.2 kb was excised from the gel, and the DNA fragments electroeluted (15). The fragments were then ligated into HindIII-SalI digested pJRD215. Kanamycin resistant transformants were screened for streptomycin sensitivity. Plasmid DNA was isolated from kanamycin-resistant, streptomycin-sensitive clones and digested with appropriate restriction enzymes to identify plasmids containing an insert having the same restriction pattern as plasmid pNZ15 (the wild-type algC gene cloned in pJRD215). One plasmid, pNZ15-58, was used as the source of the algC' gene.

For the S1 nuclease protection assay, probe labelling, hybridizations with RNA, and S1 nuclease digestions were performed according to the published procedure (24). Hybridisations were performed at 55°C overnight. Approximately 50 µg of total cellular RNA was used per reaction. Single stranded DNA was digested with 10 U of S1 nuclease for 30 min at 37°C. The 5' end(s) of the mRNA was defined by running a 6% polyacrylamide gel with the S1 nuclease protected products adjacent to the sequencing ladder produced by the dideoxy chain termination method using primer PE1 (described in Results) and single stranded DNA isolated from phage mNZ25 (see Table I).

Crude Extract Preparation and Enzyme Assays

All extract preparation procedures were carried out at 4°C. Crude extracts for β-galactosidase assays were prepared in 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM β-mercaptoethanol and 1 mM MgSO4. Extracts for PMI and PMM assays were prepared in 100 mM MOPS buffer (pH 7.0) containing 1 mM DTT and 10% glycerol (buffer A). Cells were disrupted by sonication (3 x 30 sec at 100 W). The sonicated suspensions were then centrifuged at 40,000 x g for 30 min to remove cell debris. The resulting supernatant is hereafter referred to as crude extract. No effect on PMI or PMM activity was observed when crude extracts were centrifuged at 150,000 x g for 1 h.

β-Galactosidase was assayed according to Miller (25). β-galactosidase specific activities are defined as nanomoles of o-nitrophenol produced per minute per milligram of crude extract protein at 28°C, pH 7.0.
PMI and PMM were assayed as outlined by Sa'-Correia et al. (3) with the following modifications. The assay buffer used was 100 mM MOPS (pH 7.0), the amount of each coupling enzyme used (phosphoglucose isomerase, phosphomannose isomerase, and glucose 6-phosphate dehydrogenase) was increased to 1 U, the amount of mannose 6-phosphate used in the PMI assay was increased to 30 µmol, and the reaction temperature was 30°C. One unit of PMI or PMM activity is defined as that amount which leads to reduction of 1 µmol of NADP to NADPH per minute under the specified assay conditions. Specific activities are expressed as milliunits per milligram of crude extract protein. Protein concentrations were determined by the method of Bradford (26) as outlined in the Bio-Rad Protein Assay Instruction Manual, using BSA as the reference protein for construction of standard curves.

Purification of PMM and N-Terminal Amino Acid Sequence Determination

PMM was purified for N-terminal amino acid sequence determination from IPTG-induced cultures of P. aeruginosa 8858/pNZ49.

Ion-exchange chromatography. Fifty mg of crude extract protein (prepared in buffer A as described above) in a volume of 3 ml was fractionated on a Pharmacia FPLC chromatography system using a MonoQ HR 10/10 column. The starting buffer was buffer A. The flow rate was 2 ml/min and 3 ml fractions were collected. Following sample application, unbound material was washed through the column with 40 ml of buffer A. Bound material was then eluted with 80 ml of a linear gradient of NaCl (0-0.5 M) followed by 20 ml of 1.0 M NaCl. All NaCl solutions were prepared in buffer A.

Hydroxylapatite chromatography. Fractions 28-31 (from the ion-exchange step (see Fig. 5, bottom panel) were pooled, concentrated by ultrafiltration, and passed through a column of Sephadex G-25 (Pharmacia PD-10 column) using 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 10% glycerol (buffer B) for equilibration and elution. The sample was then applied to a column of hydroxylapatite (Bio-Gel HTP) equilibrated in buffer B. The column was washed with buffer B, and bound material was eluted with a linear gradient of potassium phosphate buffer (10-400 mM, containing 1 mM DTT and 10% glycerol). The flow rate was 2 ml/min and 2 ml fractions were collected. Fractions 18-22 containing PMM activity (see Fig. 5, center panel) were pooled and concentrated to 0.3 ml by ultrafiltration.

Gel-filtration chromatography. The concentrated sample of PMM recovered from the hydroxylapatite step was fractionated by gel filtration chromatography (Pharmacia Superose 12 HR 10/30 column). The elution buffer was 10 mM potassium phosphate, pH 7.0, containing 1 mM DTT and 100 mM NaCl. The flow rate was 0.5 ml/min and 0.3 ml fractions were collected.

PMM recovered from the gel-filtration step (fraction 27, see. Fig. 5, top panel) was estimated to be about 95% pure based on SDS-PAGE (Fig. 6). This preparation was used as the starting material for N-terminal amino acid sequence determination. A 0.2 ml aliquot of this preparation was passed through an RPLC reverse-phase chromatography column (ABI RU300, C4, 2.1x30 mm). Solvents A and B were 0.1% TFA in H2O and 0.1% TFA in CH3CN. Bound material was eluted with a linear gradient (0-100% solvent B over 30 min at a flow rate of 0.5 ml/min). A sample of the major UV-absorbing material (at 215 nm) recovered from the column was subjected to SDS-PAGE analysis. Only one significant protein band was observed that corresponded to a molecular weight of about 51,000. The remainder of the collected material was lyophilized, reconstituted in TFA, and sequenced by automated Edman amino acid sequencing.

Crude extracts of E. coli AC80 and AC80/pNZ49 (prepared from IPTG-induced cells) were fractionated by ion-exchange chromatography using exactly the same procedure as outlined above for the initial purification step for the P. aeruginosa PMM.

Quantitation of Alginate Production

P. aeruginosa strains were grown as confluent lawns on PIA (containing tetracycline if needed). Cells were scraped from the plates at the indicated times and suspended in 3 ml of sterile 0.9% saline. 2 ml of the suspension was centrifuged at 27,000 x g for 30 min at 4°C to remove cells. The supernatant was carefully removed and dialyzed against distilled water. The uronic acid concentration of the dialyzed sample was then determined by the method of Knutson and Jeanes (27), using authentic alginate as a standard. The cell pellet from the original 2 ml of cell suspension was resuspended in 1 ml H2O. To 0.8 ml of the suspension, 0.2 ml of 25% trichloroacetic acid was added to lyse the cells. The sample was centrifuged for 5 min, and the supernatant was discarded. The pellet was then suspended in 0.2 ml of 50 mM potassium phosphate buffer (pH 7.0) and the protein concentration determined. Alginate production is expressed as mg alginate per mg protein from 2 ml of cell suspension.

Materials

Tryptone, yeast extract, and PIA were all Difco products and were obtained through VWR Scientific (Rochester, NY). Disodium carbenicillin was obtained from Roerig (Pfizer, Inc., New York, NY) via the University of Illinois Hospital. All other antibiotics, as well as IPTG, MOPS, DTT, alginate, substrates and coupling enzymes for PMI and PMM assays, Tris-Cl, Coomassie Brilliant Blue G, protein molecular weight standards, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). Protein assay reagent, hydroxylapatite, and agarose were from Bio-Rad (Richmond, CA). Precast SDS-PAGE gradient gels were obtained from Integrated Separation Systems (Hyde Park, MA). Pre-mixed SDS-PAGE electrophoresis tank buffer and SB-100 X-ray film were from Eastman Kodak Co. (Rochester, NY). Restriction enzymes, DNA polymerase, and dialysis membranes (12,000 molecular weight cutoff) were from Bethesda Research Laboratories (Bethesda, MD). 35S-Labelled methionine and 14C-labelled protein molecular weight standards were purchased from Amersham (Arlington Heights, IL). T4 DNA ligase and exonuclease III were from New England Biolabs (Beverly, MA) and Stratagene (La Jolla, Ca), respectively. DNA polymerase I (large fragment) and S1 nuclease were obtained from Bethesda Research Laboratories (Bethesda, MD) while SequenaseTM DNA polymerase was obtained from United States Biochemical (Cleveland, OH). Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Nucleotides used for sequencing reactions and probe labelling were purchased from Pharmacia (Piscataway, NJ). All synthetic oligonucleotides were purchased from Operon Technology (Alameda, CA).

TABLE I.

Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic[a]	alg Complementation group	Source or reference
Escherichia coli			
AC80	thr leu met hsdR hsdM		(43)
HB101	F⁻ hsdS20 (r⁻B m⁻B) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 (StrR)		(15)
JM109	Δ(pro-lac) recA1 thi-1 supE endA gyrA96 hsdR relA1 (F' traD36 proAB lacI lacZΔM15)		(21)
K-38	host strain for T7 RNA polymerase-plasmid promoter system		(16)
CSH50	str^r Pro$^-$ ara (pro-lac) strA thi		(25)
Pseudomonas aeruginosa			
8821	his-1 Alg (CF isolate)	-	(11)
8822	his-1 alg-1 (spontaneous)	-	(11)
8830	his-1 Alg (stable)	-	(11)
8890	his-1 alg61	I	(12)
8894	his-1 alg12		(12)
8887	his-1 alg60	II	(12)
8897	his-1 alg-76	III	(12)
8874	his-1 alg-74	IV	(11)
8893	his-1 alg-71		(12)
8838	his-1 alg-8	V	(11)
8846	his-1 alg-16		(11)
8857	his-1 alg-27		(11)
8835	his-1 alg-5	VI	(11)
8852	his-1 alg-22		(11) (11)
8840	his-1 alg-10	-	A. Darzins
8851	his-1 alg-21	-	(11)
8855	his-1 alg-25	-	(11)
8858	his-1 alg-28	-	(11)
8863	his-1 alg-33	-	(11)
8885	his-1 alg-55	-	(12)
8892	his-1 alg-69	-	(12)
8895	his-1 alg-73	-	(12)
8896	his-1 alg-75	-	A. Darzins
8848	his-1 alg-77	-	A. Darzins
8899	his-1 alg-78		
Plasmids			
pCP13	Km^r Tc^r IncP1 mob$^+$		(11)
pJRD215	Km^r Sm^r IncQ mob$^+$		(23)
pMMB22	Ap^r Ptac IncQ/P4 mob$^+$ tra lacI		(44)
pMMB24	Ap^r Ptac IncQ/P4 mob$^+$ tra lacI		(44)
pMMB66EH	Ap^r Ptac IncQ rrnB mob$^+$ lacI		(30)
pMMB66HE	Ap^r Ptac IncQ rrnB mob$^+$ lacI		(30)
pRK2013	Km^r ColE1 mob$^+$ tra (RK2)		(14)
pKRZ-1	Ap^r promoter probe vector containing lacZ as reporter		R. Rothmel
pUC118	Ap^r lacZ' M13 intergenic region		(45)
pUC119	Ap^r polylinker of pUC118 inverted		(45)
pGP1-2	Km^r contains RNA polymerase gene		(16)
pT7-5	Ap^r T7 promoter vector		(16)
pT7-6	Ap^r T7 promoter vector		(16)
pNZ63	Ap^r algC-lacZ transcriptional fusion		This paper
pAD4038	Ap^r Ptac-algA (in plasmid pMMB24)		(3)
pAB8	Tc^r algC cloned in pCP13 (26 kb insert)		This paper
pNZ15	Km^r algC cloned in pJRD215 (2.6 kb insert)		"
pNZ15-ΔSC	Km^r algC cloned in pJRD215 (1.8 kb insert)		"
pNZ15-ΔHE	Km^r algC cloned in pJRD215, Ptac from from pJRD215 removed (2.6 kb insert)		"
pNZ15-58	Km^r mutant algC gene (algC') cloned in pJRD215 (2.6 kb insert)		"
pNZ18	Ap^r Ptac-algC cloned in pMMB22 (2.6 kb insert)		"
pNZ48	Ap^r Ptac-algC cloned in pMMB66HE (1.8 kb insert)		"
pNZ49	Ap^r Ptac-algC cloned in pMMB66EH (1.8 kb insert, reverse orientation of pNZ48)		"
pNZ530	Ap^r Km^r algC cloned in pT7-5 (same orientation as in pNZ48)		"
pNZ631	Ap^r Km^r algC cloned in pT7-6 (same orientation as in pNZ49)		"
Phage			
M13mp18	Plac lacZ		(46)
M13mp19	M13mp18 (polylinker inverted)		(46)
mNZ25	Ap^r 1.5 kb HindIII-XhoI fragment of algC cloned in M13mp19		This study

[a] an 'r' superscript indicates resistance to Ap, Km, or Tc

RESULTS

Identification of an Alginate-negative Mutant of P. aeruginosa Lacking PMM Activity. Wang et. al. (28) showed that none of the P. aeruginosa alg genes cloned previously in this laboratory encoded PMM. Thus we devised a strategy for cloning the algC gene based on the following results. Sa'-Correia et. al. (3) showed that overexpression of the P. aeruginosa algA gene in P. aeruginosa leads not only to elevated levels of the algA gene product PMI-GMP, but also to elevated levels of PMM activity. The possibility that PMM was an activity of the hyperproduced PMI-GMP protein was eliminated however, since PMM was easily separated from PMI-GMP by ion-exchange chromatography (3). Overproduction of the E. coli monofunctional PMI (the product of the E. coli manA gene) in P. aeruginosa also led to elevated levels of PMM (3).

We exploited this inducibility of PMM to identify a mutant of P. aeruginosa that lacked PMM activity. Plasmid pAD4038 contains the P. aeruginosa algA gene cloned under control of the tac promoter in the broad host range vector pMMB24 (3). pAD4038 was introduced (by triparental mating) into several alginate-negative mutants of P. aeruginosa, and the algA gene was overexpressed by IPTG induction. Crude extracts were prepared from these cells, and the levels of PMI activity were determined. The extracts were then assayed for the concomitant increase in PMM activity that accompanies overproduction of PMI (3). Elevated levels of PMM activity were observed in all extracts except that prepared from mutant 8858 (Table II). Strains 8822/pAD4038 and 8858/pAD4038 were regrown and re-examined in a separate experiment. Both strains had the expected high levels of PMI and GMP, but again only 8822/pAD4038 had a concomitant increase in PMM activity (data not shown).

TABLE II.

Specific activities of PMI and PMM in crude extracts of alginate-negative mutants of P. aeruginosa overexpressing the P. aeruginosa algA gene (pAD4038)

Strain[a]	alg marker	Specific activity (mU/mg)	
		PMI	PMM
8821	-	91.7	53.3
8822	-	234.5	31.4
8830	-	203.6	28.3
8890	alg61	283.3	36.6
8894	alg72	289.9	32.4
8887	alg60	322.7	34.4
8897	alg76	216.7	37.9
8874	alg14	298.3	32.5
8893	alg71	187.0	32.3
8836	alg9	310.7	32.4
8846	alg18	242.9	30.4
8857	alg27	88.3	22.0
8835	alg5	217.5	32.2
8852	alg22	118.6	34.0
8840	alg10	251.2	38.9
8851	alg21	116.1	30.6
8855	alg25	137.2	34.4
8858	alg28	136.7	<1.0
8863	alg33	129.3	34.6
8885	alg55	271.1	32.9
8892	alg69	186.6	33.2
8895	alg73	58.6	26.2
8896	alg75	263.1	29.3
8898	alg77	208.6	31.3
8899	alg78	166.3	33.9

[a] All of the alginate-negative mutants were derived from the stable mucoid strain 8830 by EMS mutagenesis. The normal basal levels of PMI and PMM in mucoid strains 8821 and 8830 (i.e., in the absence of plasmid-borne alg genes) are about 0.5-1.0 mU/mg and 2.0 mU/mg, respectively (3).

TABLE III

Alginate production by P. aeruginosa strains

Strain/plasmid	mg alginate/mg protein	
	24 h	48 h
8858	0	0.01
8858/pCP13[a]	0.04	0.09
8858/pAB8	3.43	5.98
8830	4.17	7.84

[a] pCP13 is the cloning vector used to construct pAB8.

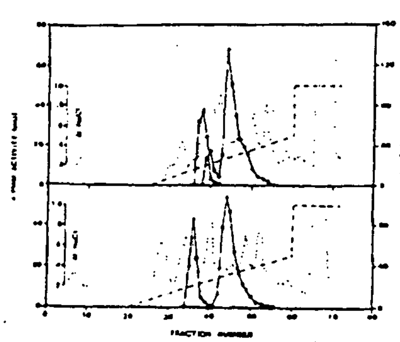

Fig. 3. Elution profiles of PMI (o) and PMM (●). Crude extracts of E. coli strains AC80 (bottom panel) and AC80/pEZ49 (+IPTG) (top panel) were fractionated by ion-exchange chromatography. The dotted and dashed lines represent A280 and the NaCl gradient, respectively.

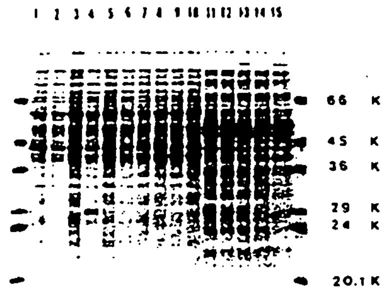

Fig. 4. SDS-PAGE of crude extracts of P. aeruginosa 8858 and E. coli AC80 harboring various plasmids containing the putative P. aeruginosa algC gene, along with the appropriate vector and uninduced controls (refer to Table IV). Lanes are numbered as follows. 1, 8858; 2, 8858 (+IPTG); 3, 8858/pMMB22; 4, 8858/pMMB22 (+IPTG); 5, 8858/pEZ18; 6, 8858/pEZ18 (+IPTG); 7, 8858/pMMB66EH; 8, 8858/pMMB66EH (+IPTG); 9, 8858/pEZ49; 10, 8858/pEZ49 (+IPTG); 11, AC80; 12, AC80/pMMB66EH; 13, AC80/pMMB66EH (+IPTG); 14, AC80/pEZ49; 15, AC80/pEZ49 (+IPTG). Molecular weight markers are shown on both sides of the gel. The migration position of PMM (lanes 5, 6, 9, and 10) is indicated by the arrow.

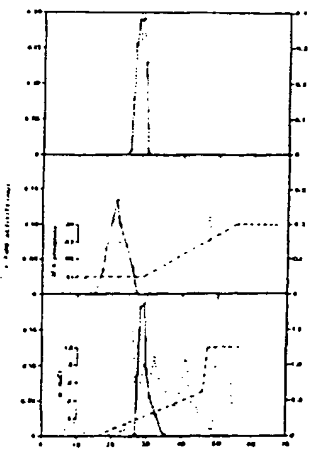

Fig. 5. Elution profiles of PMM (●) following sequential purification steps of ion-exchange chromatography (bottom panel), hydroxylapatite chromatography (center panel), and gel filtration chromatography (top panel). The dotted lines represent A280. The dashed lines in the bottom and center panels represent the NaCl (ion-exchange) and potassium phosphate (hydroxylapatite) gradients.

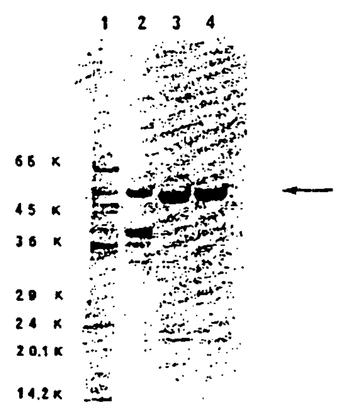

Fig. 6. SDS-PAGE of PMM preparations throughout purification steps (see Fig. 5). Lanes are numbered as follows. 1, crude extract; 2, ion-exchange; 3, hydroxylapatite; 4, gel filtration. Migration positions of molecular weight markers are shown on the left side of the gel. The arrow indicates the PMM band, migrating to a position corresponding to a molecular weight of approximately 51,000.

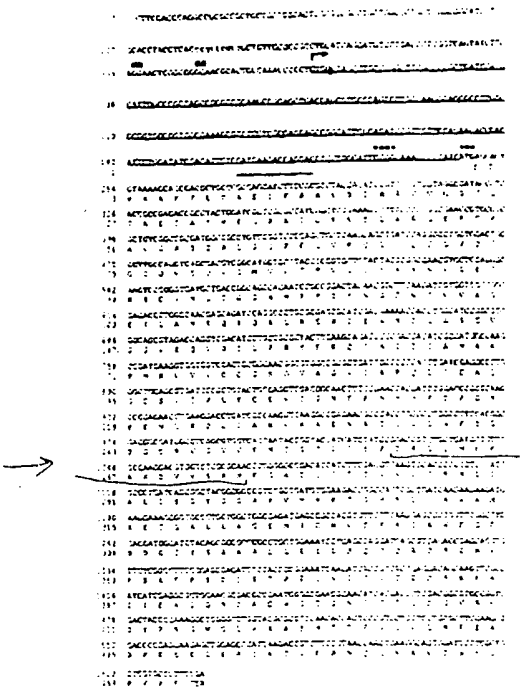

Fig. 7. Nucleotide sequence of the algC gene coding for PMM and the upstream flanking region. Nucleotides are numbered from the transcriptional start site of algC as +1. Both strands of the entire algC coding region and 421 bp upstream of the translational start site were sequenced (only the sense strand of the sequence is shown). The arrow indicates the direction of transcription and the transcriptional start site of the algC gene. The mRNA leader sequence is underlined. The translational initiation codon, ATG, is indicated by (***). The corresponding amino acid sequence is below the DNA sequence and is numbered with the translational initiation site as +1. The putative ribosome-binding site (GGAG) is indicated by (ooooo). The GC (at positions -21 and -22) and the GC (at -33 and -34) that also exist in the upstream region of the algA and algR1 genes are marked by a (#). The bases complementary to the primer used in S1 nuclease mapping are overlined. Bases shown in lower case letters (-868 to -941 are homologous to the sequence known to be the binding site for the AlgR1 protein to the upstream region of the algD gene (42).

TABLE V

Codon usage for the P. aeruginosa algC gene[a]

TTT	Phe	0	TCT	Ser	0	TAT	Tyr	1	TGT	Cys	0
TTC	Phe	17	TCC	Ser	6	TAC	Tyr	10	TGC	Cys	7
TTA	Leu	0	TCA	Ser	0	TAA	End	0	TGA	End	1
TTG	Leu	1	TCG	Ser	5	TAG	End	0	TGG	Trp	5
CTT	Leu	2	CCT	Pro	1	CAT	His	1	CGT	Arg	6
CTC	Leu	5	CCC	Pro	7	CAC	His	5	CGC	Arg	19
CTA	Leu	0	CCA	Pro	0	CAA	Gln	1	CGA	Arg	0
CTG	Leu	14	CCG	Pro	18	CAG	Gln	10	CGG	Arg	1
ATT	Ile	1	ACT	Thr	2	AAT	Asn	2	AGT	Ser	0
ATC	Ile	10	ACC	Thr	19	AAC	Asn	14	AGC	Ser	14
ATA	Ile	0	ACA	Thr	0	AAA	Lys	1	AGA	Arg	0
ATG	Met	8	ACG	Thr	1	AAG	Lys	21	AGG	Arg	0
GTT	Val	2	GCT	Ala	2	GAT	Asp	6	GGT	Gly	4
GTC	Val	18	GCC	Ala	29	GAC	Asp	27	GGC	Gly	16
GTA	Val	4	GCA	Ala	1	GAA	Glu	8	GGA	Gly	0
GTG	Val	22	GCG	Ala	3	GAG	Glu	25	GGG	Gly	3

[a] The ATG (Met) initiation codon is included

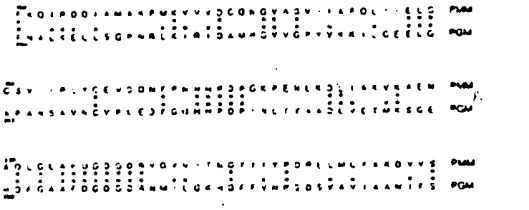

Fig. 8. Comparison of the PMM protein sequence with the PGM protein sequence. A Swiss -PROT protein data base search with the FASTP program identified a region of 112 amino acids of PMM which were homologous to PGM. The best alignment of amino acids of PMM with that of PGM are shown. Dashes represent gaps introduced to optimize alignment. Double dots indicate identical amino acids and single dots indicate conserved amino acid replacements (L=I=V=M, D=E, R=K, N=Q, S=T, and F=Y). The amino acid numbering system used is in reference to the first N-terminal amino acid position designated as +1.

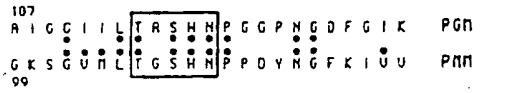

Fig. 9. Homology of the putative active site of PMM with the 21 amino acid active site region of PGM. The 5 amino acids which are critical in forming the active site of PGM (35) are boxed. Double dots represent identical amino acids and single dots indicate conserved amino acid replacements (L=I=V=M, D=E, R=K, N=Q, S=T, and F=Y). The amino acid numbering system used is in reference to the first N-terminal amino acid position designated as +1.